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ACUTE TOXICITY OF ALKYL AND PHENYLALKYLPHOSPHONATES IN THE GUINEA PIG AND RABBIT IN RELATION TO THEIR ANTICHOLINESTERASE ACTIVITY AND THEIR ENZYMATIC INACTIVATION

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Abstract—Rabbits are more susceptible than guinea pigs to the toxic action of alkyl and phenylalkylphosphonates. Rabbit red cell cholinesterase is more susceptible than guinea pig red cell cholinesterase to the inactivating action of these compounds. Multiple regression analysis revealed that the variation in the anticholinesterase activity and the ability of the phosphonates to be inactivated by enzymes in rabbit plasma accounted for 86% of the variation in toxicity of these compounds in rabbits. Only 48% of the variation in toxicity could be attributed to the anticholinesterase activity alone.

THE ACUTE intravenous toxcities of the series of *p*-nitrophenyl ethyl alkyl and phenylalkylphosphonates described previously[1, 2] were determined in both the rabbit and guinea pig. The rabbit was significantly more susceptible than the guinea pig to poisoning by these compounds. The acute toxicity of organophosphorus compounds such as these phosphonates is generally attributed to their ability to inhibit the acetyl cholinesterase present at certain physiologically important sites or 'targets'.[3, 4] We therefore investigated whether any difference exists in the ability of these same phosphonates to inactivate the red cell cholinesterase of the two species. As will be described, the red cell cholinesterase of the rabbit is distinctly more susceptible than the corresponding guinea pig enzyme to inactivation by all of the phosphonates tested. This is in agreement with the difference in susceptibility of the two species to the intravenous toxic action of the same compounds. Nevertheless, the quantitative correlation between toxic activity and anticholinesterase activity, although statistically significant, was not so large as desired. In an effort to better predict the acute toxicity, multiple regression analysis was used to provide estimates of toxicity not only from measurements of the anticholinesterase activity of the phosphonates, but also measurements of their enzymatic hydrolysis rates *in vitro* by plasma. It is generally accepted that the degree of inactivation *in vivo* is one of the greater or lesser factors which in addition to anticholinesterase activity, determines toxicity. It was hoped that the enzymatic hydrolysis rates determined previously *in vitro*[2] might serve as a measure of the rate of inactivation *in vivo*.

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MATERIALS AND METHODS

Heparinized rabbit and guinea pig blood (7 units heparin/ml blood) obtained 2 to 3 hr before each experiment was used as the source of red cell cholinesterase. The cells washed twice with 0·15 M NaCl and suspended in buffer. The red cell suspension was adjusted to have 1·0 to 1·5 $\times$ 10^9 cells per Warburg flask. The *p*-nitrophenyl ethyl phosphonates were the same as those used in work described in the preceding papers.[1, 2]

The cholinesterase activity was determined manometrically at 25° as described by Aldridge.[5] Varying concentrations of inhibitor were incubated with the cells at 25° for 15 min. At the end of this time, acetylcholine was tipped into the center compartment and the residual enzyme activity determined. The inhibitory activity of the phosphonate is expressed as the pI_{50}, which for our conditions is the negative logarithm of the concentration of inhibitor required to give 50% inhibition when inhibitor and enzyme are allowed to react for 15 min at 25°.

In a previous investigation[1] using human red cells, the cholinesterase activity was assayed by the method of Michel.[6] In order to see if the methods of Michel and Aldridge gave comparable results, the pI_{50}'s of the pentyl, octyl, and phenylbutyl-phosphonates were determined on human red cell cholinesterase by both procedures. The pI_{50}'s of the pentyl, octyl, and phenylbutylphosphonates as determined by the method of Aldridge were $6{\cdot}6_5$, $6{\cdot}7_9$, and $6{\cdot}7_9$ respectively; those obtained by the Michel technique were $6{\cdot}6_5$, $6{\cdot}7_9$, and $6{\cdot}9_5$. It was therefore presumed that the results obtained by the two methods are in fact comparable.

The hydrolysis rates of the phosphonate esters by rabbit and guinea pig plasma are those reported in the preceding paper.[2]

For measurement of the acute intravenous toxicity, groups of albino rabbits (approximately 2 kg in weight), were given single injections into the marginal ear vein, and groups of Hartley strain guinea pigs (250–300 g) were given single injections into the saphenous veins. The number of animals used for each determination varied between 30 and 50 depending on the difficulties encountered. After injection the animals were observed for pharmacological effects.

The signs of poisoning, dyspnea, tremors, miosis, paralysis, and convulsions displayed by the rabbit and guinea pig were quite similar in nature, although the order of appearance was not necessarily the same with the various compounds. The pharmacological signs observed suggested either paraylsis of the respiratory muscles or failure of the respiratory center as the cause of death. Signs of broncho-constriction were generally observed in the guinea pig but seldom in the rabbit.

The LD_{50} was calculated by the method of Bliss[7] after an observation period of 24 hr; so few deaths occurred after this time that ignoring them did not change the calculations For the rabbits the median time of death at the LD_{50} dose varied from 12 to 55 min for the compounds described here. The guinea pig survived somewhat longer, the median times of death at the LD_{50} being between 20 and 194 min.

RESULTS

Figure 1 shows for the guinea pig and the rabbit cholinesterase the pI_{50}'s of the various compounds plotted against the number of carbon atoms in the alkyl chain of the alkyl and phenylalkylphosphonates. For comparison, the pI_{50}'s obtained previously with human red cell cholinesterase[1] are also plotted in the same way.

It is evident that the human red cell cholinesterase is much more easily inactivated by these phosphonates than the cholinesterase from the rabbit which, in turn, is significantly more susceptible than the guinea pig red cell cholinesterase. The curves (Fig. 1) given by the different cholinesterases are in general parallel. However, as the number of carbons in the alkyl chain of the phenylalkylphosphonates increases from 2 to 4 there is much less of a relative increase in inhibitory activity with the guinea pig cholinesterase than with the cholinesterase of the other two species.

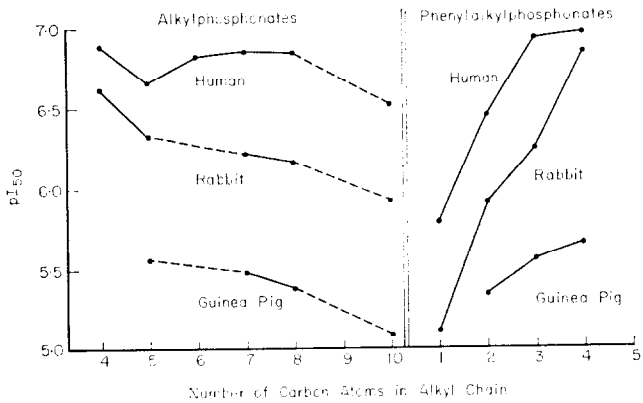

FIG. 1. Susceptibility of human, rabbit, and guinea pig red cell cholinesterase to the inhibitory action of alkyl and phenylalkylphosphonates.

Table 1 lists the intravenous toxicities (LD_{50}, μmoles/kg) and the 95% confidence limits of the twelve compounds tested in rabbits and the eight tested in guinea pigs. For comparison, the anticholinesterase activities (pI_{50}) of these compounds for rabbit and guinea pig red cell cholinesterase are also included, as well as their enzymatic hydrolysis rates (μmoles p-nitrophenol/liter liberated per 5 min) as determined with rabbit and guinea pig plasma.[2] The greater susceptibility of rabbit cholinesterase to inactivation by the phosphonates compared to the guinea pig enzyme is in accord with the significantly greater toxicity of these compounds for the rabbit (Table 1). In order to evaluate quantitatively the relationship between anticholinesterase activity and toxicity of the phosphonates in these two species the linear regression equations of log LD_{50} on pI_{50} were calculated for the data from the guinea pig and from the rabbit. These equations are given in Table 2. In both species there is a statistically significant relationship between toxicity and anticholinesterase activity (column 5, Table 2). The significant correlation coefficients, $-0{\cdot}70$ and $-0{\cdot}83$ obtained from the rabbit and guinea pig data, respectively, confirm the regression calculations.

The difference between the regression coefficient of the rabbit equation ($-0{\cdot}67$) and that of the guinea pig ($-1{\cdot}49$) was tested by student "t" test. Although large, the difference was not significant. The observed "t" based on 16 degrees of freedom was found to be 1·54 with a $P > 0{\cdot}1$. The data from the two species were used to calculate a 'combined' regression equation, Table 2. The log LD_{50} and the pI_{50} for both the rabbit and guinea pig are plotted in Fig. 2, and the regression line for the two sets of data drawn through the points. It is evident that the points from the two species appear to cluster about the same line.

TABLE 1. THE ANTICHOLINESTERASE ACTIVITY OF SOME ALKYL AND PHENYLALKYLPHOSPHONATES AS WELL AS THEIR ACUTE TOXICITY IN RABBITS AND GUINEA PIGS AND THEIR RATES OF ENZYMATIC HYDROLYSIS BY THE PLASMA OF THE TWO SPECIES

p-Nitrophenyl ethyl R phosphonate R	Rabbit				Guinea pig			
	pI_{50}	LD_{50} (μmoles/kg)	95% confidence limits (μmoles/kg)	Enzymatic hydrolysis rate*	pI_{50}	LD_{50} (μmoles/kg)	95% confidence limits (μmoles/kg)	Enyzmatic hydrolysis rate*
1. Butyl	$6{\cdot}6_1$	$0{\cdot}25_5$	0·20–0·37	$2{\cdot}7_0$				
2. Pentyl	$6{\cdot}3_2$	$0{\cdot}39_9$† $0{\cdot}49_8$†	0·33–0·53 0·40–0·60	$3{\cdot}7_5$	$5{\cdot}5_6$	$0{\cdot}99_7$	0·80– 1·23	$0{\cdot}6_7$
3. Heptyl	$6{\cdot}2_1$	$1{\cdot}67_2$	1·40–2·01	$25{\cdot}3_1$	$5{\cdot}4_8$	$4{\cdot}86_3$	3·34– 6·69	$0{\cdot}9_5$
4. Octyl	$6{\cdot}1_6$	$3{\cdot}50_0$	2·62–4·66	$37{\cdot}1_3$	$5{\cdot}3_8$	$7{\cdot}58_0$	6·69– 9·73	$1{\cdot}9_0$
5. Decyl	$5{\cdot}9_2$	$2{\cdot}34_5$	2·10–2·61	$21{\cdot}5_6$	$5{\cdot}0_8$	$7{\cdot}85_9$	4·85–12·94	$6{\cdot}6_0$
6. Benzyl	$5{\cdot}1_1$	$4{\cdot}21_1$	2·77–6·39	$1{\cdot}9_5$				
7. Phenylethyl	$5{\cdot}9_1$	$1{\cdot}04_4$	0·83–1·28	$4{\cdot}8_0$	$5{\cdot}3_3$	$2{\cdot}74_6$	1·10– 6·87	$0{\cdot}9_0$
8. Phenylpropyl	$6{\cdot}2_4$	$0{\cdot}86_0$	0·77–0·97	$6{\cdot}4_9$	$5{\cdot}5_6$	$2{\cdot}52_1$	1·89– 3·38	$0{\cdot}6_4$
9. Phenylbutyl	$6{\cdot}8_3$	$0{\cdot}24_8$	0·22–0·30	$2{\cdot}1_4$	$5{\cdot}8_6$	$0{\cdot}41_3$	0·36– 0·47	$0{\cdot}2_9$
10. Phenylethylene (styryl)	$6{\cdot}1_2$	$3{\cdot}69_3$	3·27–4·32	$10{\cdot}5_0$	$5{\cdot}1_4$	$4{\cdot}65_4$	3·66– 5·89	$5{\cdot}0_9$
11. 4-Chlorobutyl	$5{\cdot}6_9$	$0{\cdot}99_7$	0·84–2·09	$4{\cdot}1_3$				
12. 6-Chlorohexyl	$5{\cdot}8_1$	$1{\cdot}11_7$	1·00–1·23	$8{\cdot}4_4$				

* *p*-Nitrophenol, μmoles liberated/5 min/liter of plasma.

† LD_{50} of the pentylphosphonate was determined on two separate occasions.

TABLE 2. THE QUANTITATIVE RELATIONSHIP BETWEEN TOXICITY (LOG LD_{50}) AND ANTICHOLINESTERASE ACTIVITY

Species	Regression equation	± S.E. of regression coefficient	Degrees of freedom	"t"	Correlation coefficient
Rabbit	log LD_{50} + 4·109–0·6654 pI_{50}	0·218	10	3·06*	−0·70*
Guinea pig	log LD_{50} + 8·530–1·4904 pI_{50}	0·408	6	3·66*	−0·83*
Combined	log LD_{50} + 4·350–0·706 pI_{50}	0·149	18	4·75†	−0·76†

* Significant: $P < 0{\cdot}05$.
† Highly significant: $P < 0{\cdot}01$.

Although the relationship between toxicity and anticholinesterase activity is statistically significant it is apparent from Fig. 1 that gross errors would result from attempting to estimate toxicity from the anticholinesterase activity alone. In an attempt to improve this situation, the toxicity of the various compounds was related by means of multiple regression analysis to both their enzymatic hydrolysis rates and their anticholinesterase activities.

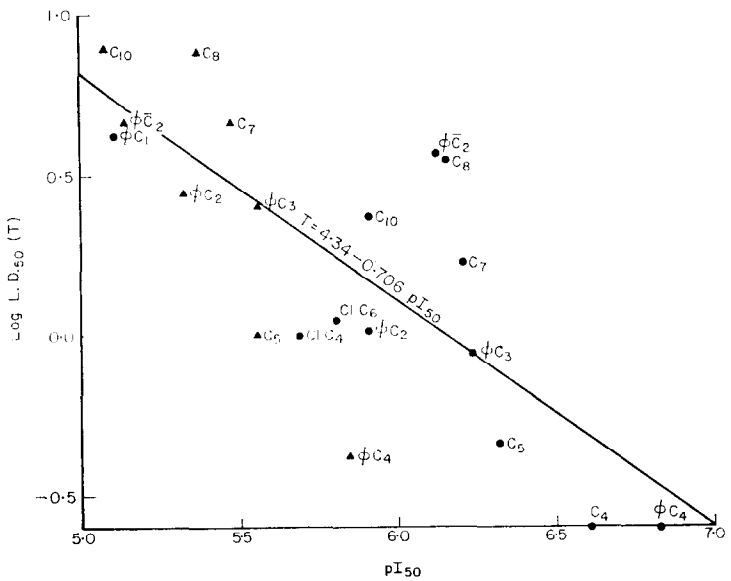

FIG. 2. Relation between acute intravenous toxicity and anticholinesterase activity in guinea pigs and rabbits: C_5 = pentylphosphonate; ϕC_1 = benzylphosphonate; ϕC_2 = styrylphosphonate; ClC_6 = chlorohexylphosphonate; etc.

The multiple regression equation relating the toxicity of the phosphonates in the rabbit to their anticholinesterase activity measured against rabbit anticholinesterase and the rate at which they are hydrolyzed by rabbit plasma is

$$\log LD_{50} = 3{\cdot}889 - 0{\cdot}7135\ pI_{50} + 0{\cdot}616 \log H$$

An analysis of variance showed that the overall regression was highly significant. The significance of the effect of anticholinesterase activity after the effect of hydrolysis rate has been removed was then tested as described by Snedecor[8] (p. 418). The same

test was then applied to learn the significance of the effect of hydrolysis rate after the effect of anticholinesterase activity was removed. The results are given in Table 3 and demonstrate that the two *in vitro* measurements taken together provide a much more satisfactory prediction of acute toxicity than either one used alone. One can obtain an estimate of the contribution of these two variables to the change in toxicity from one compound to another by calculating how much the difference in the measured pI_{50}'s and the log hydrolysis rates of the different compounds does, in fact, account for the changes in toxicity.* The pI_{50} taken by itself accounts for 48 per cent of the variation in toxicity. By adding to the pI_{50} the log hydrolysis results, as was done in the multiple regression analysis, one can account for a further 38% of the variation; i.e. the two variables between them account for 86% of the variation, leaving a maximum of 14% of the variation to be accounted for by chance errors in measurements, etc.

TABLE 3. TEST OF THE CONTRIBUTION OF LOG HYDROLYSIS AND pI_{50} TO VARIATION IN ACUTE TOXICITY IN RABBITS AFTER THE EFFECT OF THE OTHER HAS BEEN REMOVED

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
pI_{50} and log hydrolysis	2	1·72079	0·8604	28·718*
pI_{50} alone	1	1·02807	1·02807	33·431*
Log hydrolysis after pI_{50}	1	0·69272	0·69272	23·12*
pI_{50} and log hydrolysis	2	1·72079	0·8604	28·718*
log hydrolysis alone	1	1·36967	1·36967	45·716*
pI_{50} after log hydrolysis	1	0·35212	0·35212	11·754*
error	9	0·26961	0·02996	

* Highly significant: $P < 0{\cdot}01$.

1. Test for fit of multiple regression: $F = 0{\cdot}8604/0{\cdot}02996$
2. Test for effect of pI_{50} alone: $F = 1{\cdot}02807/0{\cdot}02996$
3. Test for effect of hydrolysis alone: $F = 1{\cdot}36967/0{\cdot}02996$
4. Test for effect of hydrolysis after pI_{50} removed: $F = 0{\cdot}69272/0{\cdot}02996$
5. Test for effect of pI_{50} after hydrolysis removed: $F = 0{\cdot}35212/0{\cdot}02996$

Applying the same sort of analysis to the guinea pig results, the multiple regression equation is

$$\log LD_{50} = 6{\cdot}484 - 1{\cdot}117\ pI_{50} + 0{\cdot}211 \log H$$

An analysis of variance showed that the overall regression was just barely significant at the 5% level. The contributions of hydrolysis rate and anticholinesterase activity to this regression were tested in the same way as for the rabbit data. As the results in Table 4 show, there is not only no effect of log hydrolysis rate after the effect of pI_{50} has been discounted, but the effect of the pI_{50} after the effect of log hydrolysis rate has been removed is not quite, although almost, significant statistically.

The inability to demonstrate any effect of adding a second variable is due to the

* This is done by taking the ratio of the sum of squares attributable to regression to the total sum of squares. In the regression of toxicity on pI_{50}, the sum of squares attributable to regression is 0·962; the total sum of squares is 1·990. In the multiple regression of toxicity of pI_{50} and log hydrolysis rate, the sum of squares due to regression is 1·721, and the total sum of squares is the same as above, 1·990.

very high correlation between the log hydrolysis rates and the pI_{50}'s for the eight compounds tested in the guinea pig; the correlation coefficient (R) is −0·95.* This should be compared with the negligible correlation between the independent variables for the twelve compounds tested in the rabbit (R = 0·08). Since only eight of the compounds were used in the guinea pig, it is possible that by chance eight compounds were chosen which happen to have a high correlation between pI_{50} and hydrolysis rate. It is also possible that in the guinea pig the structures of the two enzymes involved are such that a good inhibitor of one is a poor substrate of the other. Only further work would tell.

DISCUSSION

Species differences in the susceptibility of true acetyl cholinesterase to the inactivating effect of organophosphorus poisons is well recognized, e.g. the greater susceptibility of rat[9] than of horse[10] cholinesterase to inactivation by a number of different phosphates, phosphofluoridates, pyrophosphates, etc. Heath[3] (pp. 146 *et seq.*) and O'Brien[4] give other illustrations of similar species differences. The results obtained in this study provide still another, and one of the clearest instances of the effect of species on the inhibition of cholinesterase by organophosphorus poisons.

TABLE 4. TEST OF THE CONTRIBUTION OF LOG HYDROLYSIS AND pI_{50} TO VARIATION IN ACUTE TOXICITY IN GUINEA PIGS AFTER THE EFFECT OF THE OTHER HAS BEEN REMOVED

Source of variation	Degrees of freedom	Sum of squares	Mean square	F
pI_{50} and log hydrolysis	2	0·96483	0·48241	5·694*
pI_{50} alone	1	0·95826	0·95826	11·311*
Log hydrolysis after pI_{50} removed	1	0·00657	0·0065657	0·07750†
pI_{50} and log hydrolysis	2	0·96483	0·48241	5·694*
Log hydrolysis alone	1	0·91271	0·91271	10·773*
pI_{50} after log hydrolysis removed	1	0·05212	0·05212	0·615†
Error	5	0·42358	0·08472	

* Significant: $P < 0·05$.
† Not significant: $P > 0·05$.

1. Test for fit of multiple regression: $F = 0·48241/0·08472$
2. Test for effect of pI_{50} alone: $F = 0·95826/0·08472$
3. Test for effect of hydrolysis alone: $F = 0·0065657/0·08472$
4. Test for effect of hydrolysis after pI_{50} removed: $F = 0·00657/0·08472$
5. Test for effect of pI_{50} after hydrolysis removed: $F = 0·05212/0·08472$

The rank order of sensitivity of the cholinesterase to inhibition by the *p*-nitrophenyl ethyl phenylalkyl and alkylphosphonates is human > rabbit > guinea pig. What the biochemical basis is for such variation in susceptibility among the enzymes from different species is not known. These differences could arise from variation from species to species in the ease with which the phosphonates are able to combine with

* If there is, as in this case, a very high correlation between the two independent variables, making measurements on both variables is essentially equivalent to making duplicate measurements on either one. The choice of independent variable then becomes a matter of the ease of measurement and its reliability.

the active site of the enzyme, or in differences in the ability of the enzyme to be phosphorylated, or to differences in both factors.

The rank order of the ability of cholinesterase from the three species to hydrolyze acetylcholine is human > guinea pig > rabbit. This differs from the rank order of their susceptibility to inactivation by phosphonates. The significance of this difference in rank order is impossible to evaluate, since it is not known whether the differences in the cholinesterase activity of the red cells reflect differences in the concentration of the enzyme per cell in the three species or in the intrinsic activity of the enzymes themselves.

The greater sensitivity of rabbit cholinesterase to inactivation by the phosphonates compared to the guinea pig enzyme correlates very well with their greater toxicity for the rabbit. The finding that human red cell cholinesterase is the most easily inactivated of the the three tested suggests that man might be the most sensitive of the three species to the toxic action of the phosphonates. Fortunately, there are no data at present available to test this prediction.

In the rabbit, a quite good agreement was found between the experimentally observed toxicities and those predicted by the multiple regression of toxicity on enzymatic hydrolysis rates and anticholinesterase activity (multiple correlation coefficient, R = 0·96). The choice of the two independent variables, pI_{50} and log hydrolysis rates was dictated by the hypothesis that they were *in vitro* measures of at least two of the *in vivo* factors—i.e. cholinesterase inhibition and phosphonate inactivation—upon which the toxicity depended. The excellent predictive ability of these two measurements *in vitro* provides powerful evidence in support of this hypothesis but does not prove it. The alternative hypothesis, that the success was due to a happy concatenation of chance correlations—an exemplification as it were of the cautionary principle that accidents can happen in both directions—is deemed less likely, although it cannot be ruled out by the present evidence.

Acceptance of the first hypothesis does not imply that the inhibition of red cell cholinesterase is the cause of death, nor that the measurement *in vitro* of the hydrolysis rate tells us directly the degree of breakdown of these compounds *in vivo*. As Heath points out (p. 106) "Substrate specificities vary with the species from which the enzymes (cholinesterases) are taken, but not with the part of the animal from which they are taken." Grob and Harvey[11] have shown that the cholinesterase of real blood cells, brain, and muscle was inhibited to quantitatively the same extent by a number of different organophosphorus inhibitors. Thus, measurements of the susceptibility of red cell cholinesterase may be expected to provide a good estimate of the susceptibility to inactivation of the cholinesterase at the 'target' site. More direct evidence on this point as well as on the relative contributions of plasma and tissue enzymes to the inactivation of the phosphonates is undoubtedly desirable, even though the intravenous route of injection of the compounds and the high activity of the plasma enzyme would themselves suggest an important role for this enzyme in such inactivation.

In the guinea pig, the analysis (Table 4) indicates that the effect of cholinesterase inhibition alone and the rate of hydrolysis alone contribute a significant amount to the variability in toxicity. However, the high correlation between cholinesterase inhibition and phosphonate inactivation (R = −0·95) prevents us from drawing any clear-cut conclusions about the interrelationship of these two variables. Thus,

possibly because of the unfortunate choice of compounds, one can tell nothing from the data about the importance or lack of importance of each independent variable after the effect of the other has been removed. It is unfortunate that present practical difficulties prevent us from accumulating more data in the guinea pig, since the much lesser rate of hydrolysis of the phosphonates by guinea pig plasma than by rabbit plasma[2] suggests the possibility that if, in the guinea pig, enzymatic inactivation could be shown to have a significant bearing on toxicity, it should be quantitatively less important than in the rabbit.

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